Antisense treatments for biothreat agents

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Antisense oligomers (ASOs) represent a promising technology to treat viral and bacterial infections, and have already been shown to be successful against a variety of pathogens in cell culture studies and nonhuman primate models of infection. For these reasons, antisense technologies are being pursued as treatments against biothreat agents such as Ebola virus, dengue virus and Bacillus anthracis. Several generations of modified oligonucleotides have been developed to maximize nuclease resistance, target affinity, potency, cell entry, and other pharmacokinetic properties. First-generation ASOs contain phosphorothioate modifications to increase stability through nuclease resistance. Further chemical modifications in second-generation ASOs include 2'-O-methyl and 2'-O-methoxy-ethyl oligos, which increase nuclease resistance and oligo:RNA binding affinities. Third-generation ASOs contain a variety of chemical modifications that enhance stability, affinity and bioavailability. A fourth class of oligonucleotide-based compounds consists of small interfering RNAs, which have recently become widely used for gene knockdown in vitro and in vivo. This review focuses on the third-generation phosphorodiamidate morpholino oligomers, which are nonionic and contain a morpholine ring instead of a ribose, as well as phosphorodiamidate linkages in place of phosphorothioates. Multiple antisense oligomer-based therapeutics are being developed for use against biothreat agents, and antisense drugs will likely become a critical member of our arsenal in the defense against highly pathogenic, emerging or genetically engineered pathogens.

Keywords Antisense, biothreat, oligonucleotide, phosphorodiamidate morpholino, phosphorothioate, small interfering RNA

Introduction

Many viral and bacterial agents have a history of state-sponsored 'weaponization', including Marburg, Ebola, Junin, Machupo, yellow fever viruses and smallpox, as well as *Bacillus anthracis*, *Yersinia pestis* and *Francisella tularensis* [1-5]. These agents are considered serious public health threats, are handled under biosafety level (BSL)-3 or -4 conditions [6], and are listed as potential biothreat agents by the National Institute of Allergy and Infectious Diseases (*http://www3.niaid.nih.gov/Biodefense/bandc_priority.htm*). Features that characterize a pathogen as a serious bioweapon threat include high morbidity and mortality rates, the potential for person-to-person or aerosol transmission, low infective dose, stability, and the feasibility of large-scale production. Unfortunately, many of the biothreat agents are also characterized by a lack of available vaccines, prophylactics or treatments, and so developing preventatives and therapeutics against these dangerous organisms has become a high public health priority in recent years [7•].

Since the first use of antisense oligomers (ASOs) for inhibiting Rous sarcoma virus replication in cell culture [8,9], there has been a keen interest in developing ASO therapeutics. ASOs function by binding complementary sequences of RNA, and inhibiting gene expression in a rapid and highly specific manner. The manner in which they inhibit expression of the target gene has made them powerful tools to dissect critical pathways, and also as potential drugs for targeting host and pathogen genes. Here, we will provide a representative, but not exhaustive, presentation of ASOs in development as therapeutics, and provide information on advances made in the ASO field for use against biothreat agents.

Antisense oligonucleotides

First-generation antisense oligomers

Unmodified oligodeoxynucleotides are highly unstable in bodily fluids because of their rapid degradation by nucleases (Table 1). To enhance oligomer stability, replacement of a nonlinking oxygen atom with sulfur within the phosphodiester linkage of each nucleotide renders first-generation antisense phosphorothioate oligomers (PTOs) inefficient substrates for both endo- and exo-nucleases (Table 1) [10]. PTOs inhibit gene expression after binding to the target mRNA, and thereby direct the mRNA for RNase H degradation as a result of the formation of the PTO:mRNA duplex. To mediate efficient suppression of fully complementary mRNAs, optimal PTOs are 15 to 21 nucleotides long; however, transient hybridization can occur between the ASO and mRNAs with short complementary sequences, and DNA:RNA heteroduplexes as short as five nucleotides can initiate RNase H1-mediated degradation of the RNA component [11-14].

Second-generation antisense oligomers

A second-generation approach to ASO design incorporates further modifications to the inter-nucleoside ribose-phosphate linkages to improve the affinity, stability and activity of these drugs (Table 1). 2'-*O*-(2-Methoxy)ethyl (2'MOE)-modified oligonucleotide:RNA heterodimers are not substrates for cleavage by RNase H. Therefore, mRNA cleavage is limited to a short stretch of phosphorothioate nucleotides with unmodified sugars at the center of the ASO [15-17]. PTOs with 2'MOE 'wings', consisting of five 2'MOE-modified

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14. ABSTRACT
Antisense oligomers (ASOs) represent a promising technology to treat viral and bacterial infections, and have already been shown to be successful against a variety of pathogens in cell culture studies and nonhuman primate models of infection. For these reasons, antisense technologies are being pursued as treatments against biothreat agents such as Ebola virus, dengue virus and Bacillus anthracis. Several generations of modified oligonucleotides have been developed to maximize nuclease resistance, target affinity, potency, cell entry, and other pharmacokinetic properties. First-generation ASOs contain phosphorothioate modifications to increase stability through nuclease resistance. Further chemical modifications in second-generation ASOs include 2'-O-methyl and 2'-O-methoxy-ethyl oligos, which increase nuclease resistance and oligo:RNA binding affinities. Third-generation ASOs contain a variety of chemical modifications that enhance stability, affinity and bioavailability. A fourth class of oligonucleotide-based compounds consists of small interfering RNAs, which have recently become widely used for gene knockdown in vitro and in vivo. This review focuses on the third-generation phosphorodiamidate morpholino oligomers, which are nonionic and contain a morpholine ring instead of a ribose, as well as phosphorodiamidate linkages in place of phosphorothioates. Multiple antisense oligomer-based therapeutics are being developed for use against biothreat agents, and antisense drugs will likely become a critical member of our arsenal in the defense against highly pathogenic, emerging or genetically engineered pathogens.

15. SUBJECT TERMS
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Table 1. Properties of selected oligonucleotide compounds.

Antisense oligomers	Example	Chemical modifications	Net charge	Mechanism of action	Advantages	Disadvantages
Unmodified oligodeoxynuclotides	Naked DNA	None	Negative	RNase H cleavage	-	Serum half-life ~ 1 h Rapidly degraded in biological fluids by nucleases
First-generation	Phosphorothioate ODNs	Sulfur molecule replaces one of the nonbridging oxygen atoms in the phosphodiester bonds	Negative	RNase H cleavage	Serum half-life 9 to 10 h Enhanced nuclease resistance Normal Watson-Crick base pairing Activates RNase H Attractive pharmacokinetic properties Enhanced specificity of hybridization compared with unmodified ODNs	Acute toxicity resulting from activation of complement cascade Binding to proteins such as heparin-binding proteins Slightly reduced affinity toward complementary RNA molecules compared with other ODNs
Second-generation	2'-*O*-methyl and 2'-*O*-methoxy-ethyl RNA	Alkyl modifications at the 2'-position of the ribose on the nucleotide	Negative	Blocks translation (ie, steric hindrance)	Serum half-life 6 to 12 h Less toxic than phosphorothioate ODNs Slightly enhanced affinity toward complementary RNAs Can block splicing sites to increase expression of alternatively spliced protein variants	Binding to proteins such as heparin-binding proteins
Third-generation	Phosphorodiamidate morpholino oligomers	Nonionic DNA analogs with the ribose replaced with a morpholine ring and phosphorodiamidate intersubunit linkages instead of phosphodiester bonds	Neutral	Blocks translation (ie, steric hindrance)	Serum half-life 1 to 20 h Limited binding to serum proteins such as nucleic-acid-binding proteins Favorable base stacking and relatively high duplex stability Nuclease and protease resistant Limited off-target alteration of gene expression through G-quartets Limited metal chelation since there is no net charge to mediate binding No altered coagulation times Lack immune stimulation via CpG motifs of the complement cascade	Limited cellular uptake of naked PMOs Undetermined efficacious human doses against infectious diseases
RNAi	siRNAs	Double-stranded RNA	Negative	Targets RNA to RISC complex for degradation	High potent activity Chemical modifications can increase uptake Mechanisms of action are well understood	Difficult to deliver naked siRNAs into tissues May induce interferon responses Limited tolerability for subsequent chemical modifications Sensitive to nuclease degradation May require large doses for efficacy in humans

ODN oligodeoxynucleotide, **PMO** phosphorodiamidate morpholino oligomer, **RISC** RNA-induced silencing complex.

phosphorothioate-linked nucleotides at both the 5' and 3' ends, bind more strongly to RNA and deplete target mRNAs more potently than their 2' unsubstituted analogs [18-20]. Many other possible backbone modifications have been explored for RNase H-dependent ASO drugs, such as locked nucleic acid (LNA), PTO with RNase-incompetent methylphosphonate nucleotides, or 2'MOE modifications in the middle of a PTO [21-23]. Of these, 2'MOE-winged PTOs appear to be the most promising and thoroughly developed PTO-based therapeutic. In fact, the first antisense drug approved by the FDA, fomivirsen, is a 2'MOE PTO designed to treat cytomegalovirus-induced retinitis in AIDS patients [24].

Third-generation antisense oligomers

Another major class of ASO compounds, phosphorodiamidate morpholino oligonucleotides (PMOs), achieve nuclease resistance by incorporating nucleosides with a morpholino instead of ribose backbone, and also utilize uncharged internucleoside phosphorodiamidate linkages (Table 1) [25]. RNA:PMO hybrids are not generally substrates for RNase H cleavage, but suppress mRNA targets through high-affinity binding at the sites of ribosomal entry, mRNA processing, or by blocking the formation of important secondary structural elements [26-29]. Therefore, PMOs are generally designed to target important translational signals, such as the AUG start site or secondary structures immediately 5' or 3' of the AUG start site. The neutral chemistry of PMOs confers many desirable safety and pharmacokinetic properties compared with other ASOs, including avoidance of non-specific binding to host proteins and excellent resistance to nucleases and proteases (Table 1). The nonionic characteristics of PMOs have been suggested to hinder cellular uptake compared with previous-generation negatively charged ASOs. However, this aspect of PMO chemistry does not appear to affect *in vivo* efficacy and may be an artifact of cell lines, since PMOs readily enter primary cells [27]. Additionally, conjugation with specific peptide tags such as arginine-rich peptides increases PMO entry into cells [30,31••]. PMOs promise a simple method of antisense drug design, and a drug platform that is cheaper to produce than phosphorothioate-based ASO drugs [32].

RNA interference

In recent years, small interfering RNA (siRNA) has been developed as a powerful gene silencing platform both *in vitro* and *in vivo* (Table 1) [33]. The RNA interference pathway through which siRNAs are recognized in the cytoplasm, cut into short fragments, and catalytically utilized by cells to degrade other complementary transcripts in the nucleus, is well understood and described elsewhere [34-36]. Rapid tail vein injections of unmodified siRNA in mice have proved efficacious [37,38]. However, other routes of administration have not been as successful *in vivo*, seemingly as a result of rapid degradation of unmodified siRNA molecules in mammals [39]. However, introducing chemical modifications at the 3' ends of both siRNA strands confers *in vivo* stability [40••]. Ongoing clinical trials will shed light on the potential of siRNAs for use as therapeutics.

Potential of antisense oligonucleotides as therapeutics

The use of ASOs as therapeutics relies upon specific hybridization of the oligomers to the target mRNA, the capacity of the ASO:mRNA complex to inhibit gene expression, and sufficient biological stability and activity of the ASOs *in vivo*. While many of the lead candidate ASO-based therapeutics are highly specific and effective in inhibiting gene expression of the target of interest *in vitro*, there are many potential barriers toward *in vivo* efficacy for this class of therapeutics. After *in vivo* administration, the ASO must penetrate multiple barriers, gain entry to the desired area of the body, enter the target cells, and localize to the intracellular compartment necessary for inhibiting gene expression. Throughout this process, the ASO must remain intact by avoiding nuclease degradation and not be cleared from the body before gene knockdown occurs. Furthermore, especially for bacterial pathogens, the ASO may have to traverse within the pathogen to target the pathogenic mRNAs.

Among the ASOs, the pharmacokinetics of PTOs and modified PTOs in humans are fairly well understood. Intravenously administered PTO and 2'MOE-modified phosphorothioate (PS) oligonucleotides distribute primarily to highly perfused tissues (the liver, kidneys, bone marrow, skin and skeletal muscles), with only about 1% of the oligomers remaining available in the plasma [41-44]. Intravenous administration of PTO compounds may be limited by distribution mainly to highly vascularized tissues due to promiscuous interactions with serum proteins via their polyanionic backbone [45]. Encouragingly, administering PTOs subcutaneously rather than intravenously results in a much higher plasma concentration [46]. Three days after subcutaneous administration of PTOs to humans, up to 30% of PTO remains in the plasma. For this reason, subcutaneous administration may become the preferred method for delivering PTO-based drugs [46]. First- and second-generation PTOs have shown promise *in vivo* against fulminant hepatitis and hepatitis C virus infections [20,47], as well as non-liver diseases such as cancers, through the inhibition of various targets, including protein kinase C-α (aprinocarsen), Bcl-2 (oblimersen; Genta Inc), Raf kinase (ISIS-5132), Ha-ras (ISIS-2503), RIα regulatory subunit of cAMP-dependent protein kinase type I (Gem-231) and clusterin (OGX-011; OncoGenex Technologies Inc) [48]. However, multiple reactions to PTOs during human clinical trials have been observed, including pronounced cardiovascular responses, activation of the complement cascade, thrombocytopenia, hyperglycemia and hypotension [49].

PMOs have been safely employed in more than 12 clinical trials involving over 300 individuals without adverse drug-related events occurring [49-51]. These initial clinical studies have also demonstrated pharmacokinetic elimination half-lives in humans of 1 to 20 h [49]. The potential toxicity of PMOs has been evaluated in mice, rats and nonhuman primates, and the only noted toxicity to date is the transient

appearance of basophilic granules in macrophages, Kupfer cells and other phagocytic cells in lymph nodes when PMOs are administered for at least 14 days [27]. PMO sequences have also been evaluated in Good Laboratory Practice genotoxicity batteries with no observed mutagenesis [27].

In vivo studies of siRNA biodistribution in mice show a similar profile to other classes of oligonucleotide drugs [52]. However, to date, there is only one report of a siRNA-based drug being used in humans; Sirna-027 (Sirna Therapeutics Inc/Allergan Inc) targets vascular endothelial growth factor receptor-1, a receptor involved in angiogenesis. The company aims to use this drug to treat age-related macular degeneration [53•]. Encouragingly, in six of seven patients treated with a single, intravitreal dose of Sirna-027, there was a reduction in retinal thickness [53•]. However, because the siRNAs were directly injected into the eye, it may be difficult to extrapolate these findings to systemic siRNA treatment of humans.

Antisense treatment of viral infection

At the beginning of antisense research, the first investigators tested whether Rous-sarcoma-virus-complementary oligonucleotides could interfere with viral amplification [8,9]. Since then, gene knockdown using ASOs against a variety of agents has been demonstrated to be quite effective in squelching viral amplification (Table 2). More recently, interest in using ASO-based approaches against biothreat agents has peaked, and several new reports provide evidence that ASO therapeutic candidates will be effective for treating acute viral diseases such as Ebola and dengue (Table 2).

Table 2. Summary of studies of antisense efficacy against viral agents.

Virus	Antisense type	Model inhibition demonstrated	References
Avian myeloblastosis virus	Unmodified and modified antisense oligodeoxynucleotides	*In vitro*	[83,84]
Calcivirus	PMO	Cell culture	[85]
Coronavirus (including SARS)	PMO siRNA	Cell culture, nonhuman primates	[28,86,87•]
Encephalomyocarditis virus	Antisense oligonucleotides	Cell-free rabbit reticulocyte lysates	[88]
Ebola virus	PMO	*In vitro*, cell culture, rodents, nonhuman primates	[60,61]
Epstein-Barr virus	PTO	Cell culture	[89,90]
Equine arteritis virus	PMO	*In vitro*, cell culture	[91]
Flaviviruses (ie, dengue and West Nile viruses)	PMO	*In vitro*, cell culture	[65-68,69•]
Friend retrovirus	Oligonucleotides	Cell culture	[92]
HBV	Unmodified or chimeric DNA-RNA PTOs siRNA	*In vitro*	[93-96]
HCV	PNAs and LNAs 2'-*O*-methyloligoribonucleotides	*In vitro*	[97,98]
HIV	LNA/DNA chimeric oligomers 2'-*O*-methyloligoribonucleotides Methylphosphonate-phosphodiester hybrid oligonucleotides Phosphate-methylated DNA Unconjugated and conjugated PTOs Methylphosphonate oligodeoxynucleotides	*In vitro*, cell culture	[10,93,99-114]
HSV	Polyalkylating single-stranded oligomers Unconjugated and conjugated PTOs 2'-*O*-methyloligoribonucleoside methylphosphonate oligos	Cell culture	[115-119]
Infectious hematopoietic necrosis virus	PMO	Cell culture	[120]
Lentiviruses	Phosphorothioate ON	Cell culture	[121]
Mouse leukemia virus	Modified and unmodified PTOs	Cell culture and mice	[122]
Respiratory syncytial virus	Modified and unmodified PTOs	*In vitro*, cell culture, non-human primates	[123••]
Retroviruses	PTO	Cell culture	[121]
Tobacco mosaic virus	Complementary ODN	*In vitro*	[124]
Vesicular stomatitis virus	Unconjugated and conjugated PTOs Methylphosphonate oligonucleotides	Cell culture	[117,125-127]
Vesiviruses	PMO	Cell culture	[128•]

HBV hepatitis B virus, **HCV** hepatitis C virus, **HSV** herpes simplex virus, **LNA** locked nucleic acid, **ODN** oligodeoxynucleotide, **ON** oligonucleotide, **PMO** phosphorodiamidate morpholino oligomer, **PNA** peptide nucleic acid, **PTO** phosphorothioate oligomers.

Development of antisense treatments for filovirus infections

The filoviruses, Ebola (EBOV) and Marburg (MARV), are highly virulent and cause acute, severe hemorrhagic fever in humans and nonhuman primates. Although filovirus infection most often leads to death, it is not uniformly lethal in humans. Numerous studies clearly demonstrate that the survival of an infected individual or animal is critically dependent on the ability of the host's innate and adaptive immune responses to control rapid viral growth [54-57]. Thus, host- or virus-targeted therapeutics that could curb filovirus replication until generation of an effective host immune response may prove efficacious in prevention of lethal disease. MARV and EBOV have a 19-Kb, negative-sense, single-stranded RNA genome that encodes for nucleoprotein (NP), glycoprotein (GP), four viral proteins (VP), and RNA-dependent RNA polymerase (L protein) [58]. Successful transcription and replication of the viral genome depends on coordinated action of L, NP, VP30 and VP35 proteins. The VP24 and VP40 proteins coordinate budding of the virions from the cell, where the GP is incorporated into the membrane during capsid formation [58].

Currently, there are no available vaccines or therapeutics for treating EBOV infections in humans. Although many different strategies have been attempted, only the administration of a recombinant tissue Factor VIIa inhibitor is protective in 33% of treated nonhuman primates [59]. Recently, Warfield *et al* and Enterlein *et al* described successful strategies for interfering with EBOV infection *in vitro* and *in vivo* by using untagged and peptide-tagged PMOs [60••,61]. Therapeutic administration of a single untagged or peptide-tagged PMO targeting the viral VP35 mRNA significantly reduced viremia and resulted in 100% survival in EBOV-infected mice [60••,61]. A combination of EBOV-specific, untagged PMOs targeting VP24, VP35 and RNA polymerase L protected mice and guinea pigs in both pretreatment and post-exposure therapeutic regimens [60••]. In a prophylactic proof-of-principal trial, the PMOs also protected 75% of rhesus macaques from lethal EBOV infection [60••]. Screening studies for efficacious PMOs against MARV have yielded positive results in the guinea pig model and, to date, we have identified more than five compounds that resulted in high levels of survival in initial studies [Warfield KL, unpublished data].

siRNAs are also expected to provide robust treatment options for hemorrhagic fevers because siRNAs act in a self-amplifying enzymatic manner. The inhibition of filovirus amplification by siRNAs was demonstrated by targeting VP30, VP35, and the NP of MARV [62]. Recently, siRNAs targeting the EBOV L protein were shown to inhibit EBOV replication *in vitro*, as well as providing post-exposure protection to guinea pigs infected with EBOV [63]. Future studies in nonhuman primates may reveal the therapeutic potential of siRNA treatment of humans with filovirus infections.

Antisense treatment for flavivirus infections

The family *Flaviviridae* includes highly pathogenic viruses such as West Nile, yellow fever, dengue and Omsk hemorrhagic fever. The flaviviruses are encoded by a single-stranded, positive-sense RNA genome, and are characterized by an isometric, enveloped virion. Dengue virus is the only flavivirus considered a Category A biodefense threat by the National Institute of Allergy and Infectious Diseases (*http://www3.niaid.nih.gov/biodefense/bandc_priority.htm*). However, it was excluded as a biowarfare threat by the American Medical Association Consensus group because it is not transmissible as a small-particle aerosol, and primary dengue infections are only rarely fatal [1]. Dengue virus causes 100 million cases of dengue fever and > 500,000 cases of hemorrhagic fever in humans each year in more than 112 countries [6,64]. Dengue-virus-infected patients present with a variety of symptoms from undifferentiated fever, hemorrhagic fever, or a 'shock-like' syndrome.

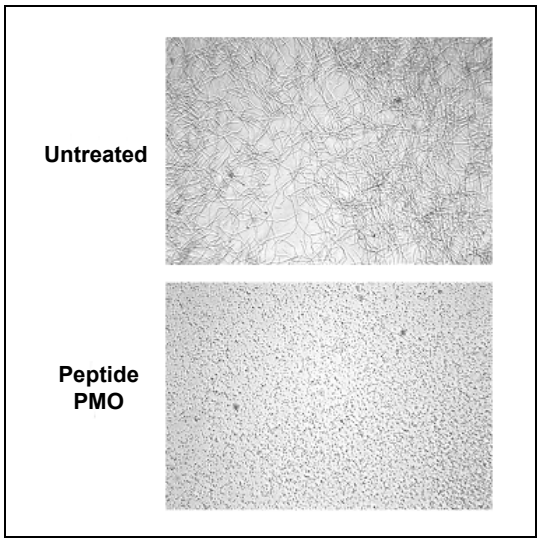
A series of peptide-conjugated (P-) PMOs have been screened and several are highly effective at inhibiting dengue viral replication by targeting RNA replication, translation of viral proteins, or both [65,66] Among the PMOs tested is the 3'-cyclization sequence (3'CS), which is designed to bind to a perfectly conserved sequence at the 3' end of the viral genome required for circularization and replication of the dengue virus genome [67,68]. Encouragingly, the 3'CS P-PMO was able to effectively reduce viral titers to nearly undetectable levels for all four serotypes [65]. Other PMOs targeting a 5'-stem-loop (5'SL) and a 3'-stem-loop (3'SL) were also effective in inhibiting viral replication [65,66]. These studies are informative both from a therapeutic design standpoint, and in revealing portions of the flavivirus genome that regulate dengue virus replication. PMOs are also effective in viral suppression of West Nile virus, another flavivirus, by blocking viral translation and RNA replication [69•].

Antisense molecules as antibacterials

Because of the emergence of antibiotic-resistant strains of bacteria, it is imperative that novel antibacterials are developed, especially ones that are effective against a broad range of bacterial agents. To date, the development of a new chemical class of antisense antibacterials is still in its infancy but has shown some promise against certain bacterial agents [70,71]. Synthetic ASOs, called peptide nucleic acids (PNA) can inhibit the expression of *Escherichia coli* bacterial genes both *in vitro* and directly in the organism [72,73]. However, the entry of PNA is inefficient because of the lipopolysaccharide layer of the outer membrane of Gram-negative bacteria [74]. Antisense PMOs have limited cellular uptake in *E coli* but can efficiently inhibit targeted gene expression in mutant *E coli* cells that have a leaky outer membrane [75]. The feasibility of using ASOs as antimicrobial agents against Gram-positive bacteria was recently demonstrated for the infectious agent *Mycobacterium tuberculosis*. Targeted knockdown of glutamine synthetase mRNA by using phosphorothioate-modified antisense oligodeoxyribonucleotides modestly inhibited glutamine synthetase activity, formation of the cell wall structure, and bacterial replication [76]. In search of more promising targets of *M tuberculosis*, PTOs targeted against the 30/32-kDa mycolyl transferase complex showed increased inhibition of bacterial growth [77].

A major limitation of ASOs and their derivatives as gene-silencing agents is their poor uptake by target organisms. Studies have shown that altering the length and target position of antisense PMOs can enhance uptake and inhibit gene-specific expression in *E coli* [78]. Shorter PMOs (approximately 9 to 12 bases in length) effectively inhibit target gene expression in *E coli* culture, while longer PMOs (~ 20 bases) showed better inhibitory activity in cell-free expression systems than in culture [78]. A short, 11-base PMO targeted to an *E coli* essential gene relating to phospholipid biosynthesis (acpP) inhibited bacterial growth in culture and in infected mice [70]. Efficient delivery into cells can also be enhanced by conjugating synthetic oligonucleotides with peptides [31••,79,80]. Coupling peptides to PNA enhanced the entry of the antisense molecules and reduced expression of the bacterial target genes both in *E coli* [81] and *Staphylococcus aureus* [82]. Peptide-tagged PMOs can also efficiently inhibit bacterial growth in pure and infected cultures [75]. In a recent study, we observed that peptide-tagged PMOs could inhibit the outgrowth of Sterne strain of *B anthracis* (Figure 1) [Panchal RC, *et al*, unpublished data]. Studies are ongoing to determine the specificity of the target PMOs and their effect on the virulent Ames strain of *B anthracis*. While the application of antisense antibiotics to the treatment of bacterial infections looks promising, a number of issues, such as specificity, optimal size, uptake, mechanism of action, and their efficacy in animal models remain to be addressed.

Figure 1. Peptide-tagged PMOs inhibit outgrowth of *B anthracis*.



An arginine-rich peptide-tagged PMO was incubated with *B anthracis* (Sterne strain) and growth of the bacteria was assessed by light microscopy after 4 h.

Host-directed antisense-based therapeutics against biothreat viral agents

The explosion of discoveries in the area of cellular machineries and signal-transduction pathways in the past decade has had an enormous impact on medicine. Several new FDA-approved anticancer drugs (imatinib, erlotinib, gefitinib, trastuzumab and rituximab) have emerged from these studies, underscoring the importance of a deep understanding of cellular pathways for countermeasure development. While cellular pathways involved in tumorigenesis have been extensively studied, our understanding of the host pathways in the pathogenesis of viruses is still lagging behind. Understanding the cellular machineries involved in the entry, uncoating, replication, assembly and budding of viruses, as well as delineating the signaling pathways governing the genesis of the innate immune response to pathogens or subversion of such pathways by viruses, will be key to devising such novel host-targeted therapeutics. A major challenge in studying signaling pathways, in particular in complex systems such as host-pathogen interactions, is the concurrent activation or modulation of multiple cascades and cross-talk between individual pathways. In this regard gene knockdown technologies will be invaluable as a therapeutic option as well as a tool for delineating complex cellular pathways.

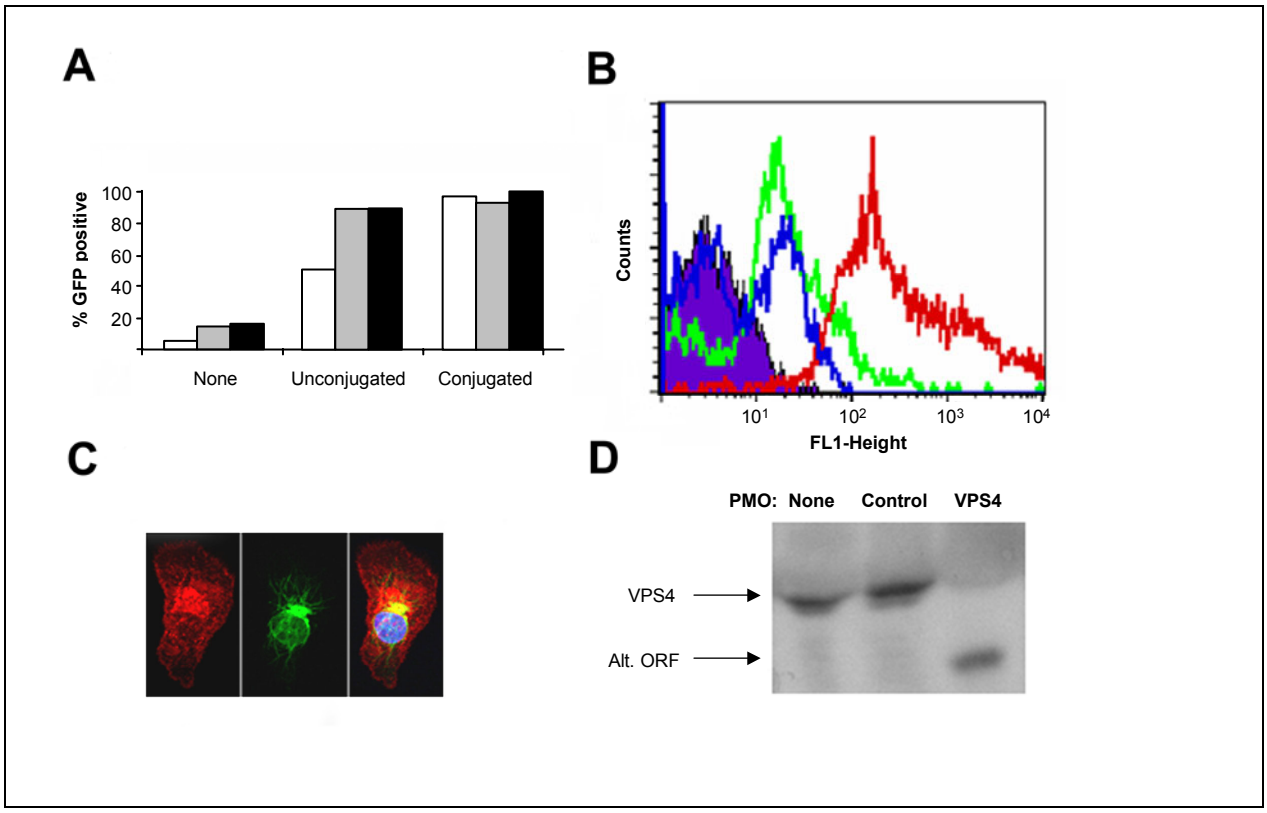
PMO oligonucleotides are prime candidates for such applications due to their ability to effectively downregulate the expression of cellular genes. Our studies show that PMOs can readily enter primary human cells such as dendritic cells, monocytes and natural killer cells (Figure 2A to C). We were able to effectively block the expression of several cellular genes by using PMOs, including vacuolar protein sorting (VPS)-4 (Figure 2D). Given the demonstrated efficacy of PMOs as gene-targeted drugs in higher animals [60••], this class of compounds represents a promising approach for developing host-targeted, broad-spectrum antiviral therapeutics. One can envision a variety of cellular targets for PMO antiviral therapeutics, including virus receptors, proteolytic enzymes involved in budding and the release of virions from infected cells, cellular signaling molecules, and components of endocytic and vacuolar protein sorting machinery.

PMOs can also be used to delineate complex signaling pathways involved in host-pathogen interactions. Combined with global systems biology-based approaches, specific gene knockdowns using PMOs will allow us to closely examine complex and interconnected biological pathways and to obtain a global view of the cellular processes during host-pathogen interactions. Information extracted from such studies can be fed into bioinformatics and computational tools to create preliminary models of combined pathogen and host response systems or pathways to distill common pathogenic and protective mechanisms. These models can then be exploited to design novel therapeutic modalities for broad-spectrum antivirals.

Conclusion

Currently, multiple viral and bacterial agents represent the most likely bioterrorism or biowarfare threats by intentional transmission or natural outbreaks of disease. Furthermore, emerging and dangerous viral and bacterial infections continue to appear throughout the world, and pose legitimate worldwide health threats. While vaccination is invaluable as a defensive resource, prophylactic measures may encounter public resistance and may not meet the risk-to-

Figure 2. Entry of PMOs into human cells.



(**A**) HeLa cells were treated with 2 μM fluorescein-tagged PMO, with or without peptide conjugation, and analyzed by flow cytometry after 2 h (white bars), 24 h (grey bars), or 48 h (black bars). (**B**) Entry of fluorescein-tagged PMOs into human dendritic cells, measured by flow cytometry as the fluorescence intensities (FL1-Height) of cells per microliter (Counts): filled trace = medium control; green trace = 2 h arginine-rich peptide-tagged PMO; red trace = 24 h arginine-rich peptide-tagged PMO; blue trace = 24 h unconjugated PMO. (**C**) Uptake of PMO in dendritic cell visualized by confocal microscopy: red, golgi and endoplasmic reticulum staining; green, PMO; yellow, merge (colocalization); blue, nuclear staining. (**D**) Western blot demonstrating that treating dendritic cells with a PMO targeted against human VPS4 gene, but not a control PMO, completely blocked VPS4A expression. Interestingly, this treatment forced the ribosomes to initiate transcription from a downstream in-frame ATG site, creating a potential dominant negative. Lower band, alternate open reading frame (Alt. ORF). Inhibition was seen as early as 24 h post-treatment and lasted for 72 h.

benefit ratio for vaccinating healthy individuals. Therefore, the most effective means of saving lives in case of an attack with a biological agent is a variety of therapeutic drugs for treating potentially exposed or ill patients in concert with prophylactic drugs and vaccines for unexposed or recently exposed populations. With the availability of rapid diagnostics and sequencing of biological agents, the causative agents in intentional or natural outbreaks of disease should be readily identified and antisense drugs could be quickly designed, if they are not already available. However, as viruses have multiple genes, a number of target sequences for each gene must be screened to identify an effective inhibitor. Therefore, the process of designing an effective antisense-based antiviral may involve the synthesis and screening of many candidates. Based on the current lack of therapeutics for most of the biothreat agents, antisense oligomer-based drugs represent one of the most efficient means of addressing emerging infectious diseases in a timely manner. The promise of specific and relatively safe therapeutics based on antisense technologies has led to recent advancements for developing treatments for diseases caused by biothreat pathogens, including Ebola and dengue viruses, as well as highly virulent bacterial agents.

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